Identification of the milk fat globule membrane proteins. II. Isolation of major proteins from electrophoretic gels and comparison of their amino acid compositions

The fat globule membranes of milk are derived from the apical plasma membrane of the mammary secretory cells. The nature of the membrane proteins, as isolated from cows' milk, has been studied by the use of discontinuous and continuous SDS-gel electrophoresis. Six methods of preparation of milk fat globule membrane suggested by various authors were tested; gel electrophoresis showed that five major bands were present, independent of the method of preparation. The apparent molecular masses of these proteins as determined on SDS-gels (15% T) were 167, 142, 64, 49 and 46 kDa, respectively. The 167 kDa band stained only with periodic acid-Schiff reagent, while the 142 kDa band stained only with Coomassie blue; the last three bands stained with both. Delipidated membranes were extracted stepwise with water, 0.02 M NaCl and 0.6 M NaCl. The 64 kDa band appears to be nearly insoluble, while the bands of 142, 49 and 46 kDa are fractionated by this procedure. The resolution of all of these proteins by electrophoresis was superior to that achieved by molecular sieve chromatography, and so electrophoretic extraction was used to isolate the major proteins. Dansyl chloride derived proteins were used as markers. Amino acid compositions of the recovered proteins were obtained and are compared.

Introduction

In lactating mammary gland, when milk is secreted by the mammary epithelial cells, the lipid globules carry with them the apical plasma membrane [1]. Analyses of milk fat globule membrane show it to be similar in composition to typical plasma membrane with regard to its marker enzymes and its content of phospholipid, glycolipid, cholesterol and protein [1]. Thus, milk fat globule membrane is an example of apical plasma membrane. The structure of all membranes is thought to be the result of the properties of their constituent proteins and lipids. Several investigators [2–7] employing different methods of preparation have used sodium dodecyl sulfate gel electrophoresis to study the protein distribution of milk fat globule membranes. Correlations among these studies have been poor, and since the method of preparation may influence the protein distribution, we decided to determine the major bands common to all preparations and to purify these bands and characterize them with regard to their amino acid compositions.

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Materials and Methods

Preparation of whole fraction of the milk fat globule membrane proteins by various methods. Fresh warm raw milk was obtained from Jersey cows. Phenylmethylsulfonylfluoride (0.25 g/l milk) was used to inhibit proteolysis [8,9]. Within 1 h after milking the sample was divided into six 250 ml lots and cream was collected by centrifugation at $5000 \times g$ for 15 min at 25°C. The six selected methods of preparation of fat globule membrane are shown in Table I. Each cream aliquot was washed three times, and gently resuspended with one to two strokes in a tissue homogenizer in a final volume of 25 ml. Cream samples were chilled at 5°C overnight and then churned with a Polytron ST-20 * until fat and sera were separated. They were warmed to 40°C and then centrifuged at $100\,000 \times g$ for 1 h at 37°C. The pellets which contained crude membrane were washed again with the corresponding buffer by homogenization and centrifugation; 750 µl of protein solvent (0.166 M Tris/1 mM EDTA (pH 8.0)) was added to each membrane preparation and the mixture was sonicated.

Preparation of the milk fat globule membrane proteins for electrophoresis and extraction. Delipidated proteins of milk fat globule membrane were prepared from fresh warm milk by the method of Herald and Brunner [6], except that water-washed cream was churned at room temperature with a Polytron ST-20. 5 vol. of deionized water were added to the delipidated protein suspension, which was then stirred for 1 h and then centrifuged 1 h at $25\,000 \times g$ and 5°C. The water-soluble fraction was collected; the pellet was then extracted sequentially with 0.02 M NaCl and 0.6 M NaCl. The proteins not extracted are termed the insoluble fraction. All fractions were exhaustively dialyzed and lyophilized.

Preparation of fluorescent milk fat globule membrane proteins for slab gel monitoring. Fluorescent proteins were prepared by reacting each major salt-soluble or insoluble milk fat globule membrane fraction with dansyl chloride in a mildly alkaline SDS system [10]; these samples were prepared for gel electrophoresis in the same manner as the other membrane and marker proteins [11].

Polyacrylamide gel electrophoresis 1. Membrane proteins were examined by polyacrylamide gel electrophoresis, using the discontinuous system described by Laemmli [12], which was modified for an E-C Vertical Slab Gel Apparatus [11]. The stacking and separating gels were prepared from 4% and 8.5% or 15% acrylamide (%T), respectively, and crosslinked with bisacrylamide (2.67% C). The gels were run for 5 h for 8.5% gel and 12 h for 15% gel. The gels were stained for protein with Coomassie blue and for glycoproteins by the method of Kapitany and Zebrowski [13]. Slab gels must be rinsed thoroughly with 15% acetic acid before staining with Schiff's reagent to prevent a high purple background.

Molecular masses of the proteins of the milk fat globule membrane were estimated by the mobility procedure of Weber and Osborn [14].

Protein components were isolated by preparative gel electrophoresis. A three-place slot former in which the end slots were 1 cm wide and the middle slot, 7.5 cm wide, was used. Appropriate dansylated fractions prepared as described above were placed in each end slot and visualized with ultraviolet light to allow excision of the unlabeled proteins run in the center slot.

Polyacrylamide gel electrophoresis 2. Membrane proteins were examined and isolated by sodium

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE I

METHODS OF PREPARATION OF MILK FAT GLOBULE MEMBRANES

No.	Buffer	Authors reporting (Ref.)
1	0.1 M imidazole-HCl (pH 7)/ 2 mM $MgCl_2$/0.25 M sucrose	Kobylka and Carraway [2]
2	0.15 M sodium phosphate (pH 7) 0.15 M NaCl/0.25 M sucrose	Anderson and Cheeseman [3]
3	10 mM Tris-HCl (pH 7.5)/ 1 mM $MgCl_2$/0.28 M sucrose	Mather and Keenan [4]
4	50 mM sodium phosphate (pH 6.8)/0.15 M NaCl	Nielsen and Bjerrum [5]
5	deionized water	Herald and Brunner [6]
6	10 mM Tris-HCl (pH 7.4)/ 2 mM $MgCl_2$/0.15 M NaCl	Jarasch et al. [7]

dodecyl sulfate polyacrylamide gel electrophoresis [15] in the continuous system previously described, using Cyanogum-41 as the gel medium.

Gel elution. The Canalco Prep-Disc apparatus was modified for the recovery of proteins from polyacrylamide gels. A sample cup fitted with a dialysis membrane was designed to attach to the bottom of PD-2/150 gel column of the Canalco apparatus (the parts were from a Sephadex SR-25 sample cup). Plastic gel support screens and O-rings were used to keep the gel in place. The reservoirs and sample cup were filled with the same pH 9.2 buffer used for electrophoresis system 2 in order to avoid glycine in the buffer. The positive electrode was placed in the lower reservoir so that the SDS-protein migrated toward the dialysis membrane from the gel. A constant voltage (350 V) was applied to this system for approx. 3 h; the system drew between 20 and 35 mA. After the run, the sample cup was removed from the apparatus and the protein removed by a syringe fitted with plastic tubing. The samples were dialyzed and lyophilized.

Gel chromatography. Sephacryl S-200 which was equilibrated with 0.083 M Tris (pH 8.5)/0.1% SDS/1 mM dithiothreitol was poured into a 2.6 × 40 cm column. Chromatography was carried out as previously described [15].

Amino acid analysis. The amino acid analyses of the milk fat globule membrane proteins were performed on a Beckman 119 CL amino acid analyzer. Protein samples were hydrolyzed at 110°C for 24 h with 5.7 M HCl containing phenol (0.05%) in sealed evacuated tubes. Statistical analysis of the amino acid composition data was carried out on the SAS system using the GLM procedure [16].

Protein analysis. Protein was determined by the Coomassie blue method for protein assay [17], using bovine serum albumin as a standard.

Results and Discussion

Comparison of methods of preparation

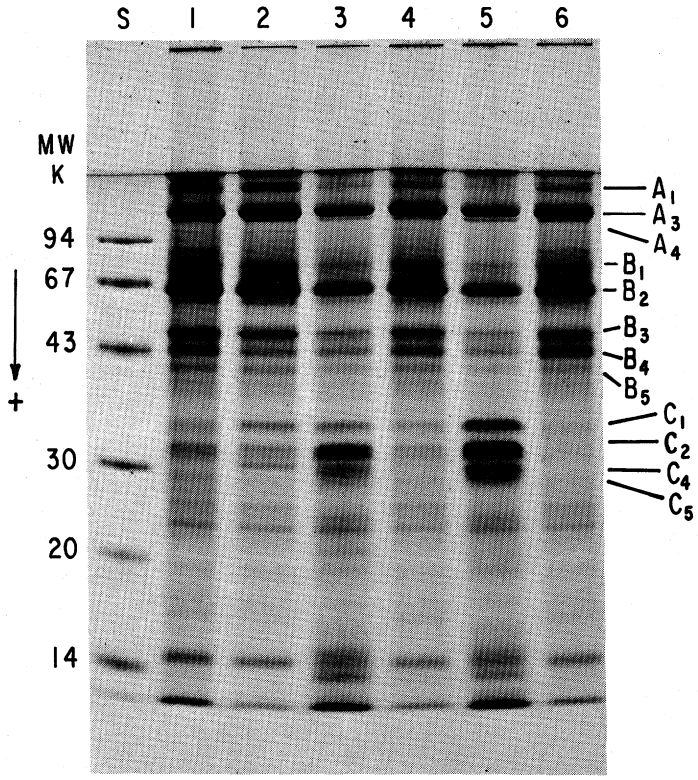
The fat globule membranes were prepared using six different buffer solutions [2–7] for washing cream (Table I). Fig. 1 illustrates how protein yields and distributions are influenced by the washing method. It has been suggested [18] that the proteins of the milk fat globule membrane can be classified on the basis of their SDS electrophoretic mobilities. Inspection of Fig. 1 indicates three gaps, which occur at approx. 94, 38 and 20 kDa. Therefore, any protein which is located in a region between the stacking-separating gel interface and 94 kDa is considered to be in zone A. Likewise, the region between 94 and 38 kDa is zone B, and the region between 38 and 14 kDa is zone C. The individual band is further identified by including its apparent molecular mass in kilodaltons as shown in Table II, e.g., the band labeled A_3 is A-142. In Fig. 1 the C bands and those under 14 kDa are greatly reduced in methods 2, 4, and 6. Table I shows that all three of these preparations have one reagent in common, 0.15 NaCl. For the six methods tested, gel electrophoresis (Fig. 1) showed that the presence of major bands in the A and B zones were independent of the method of preparation. The intensity of those bands varied due to the presence of other bands, especially in zone C, since the total protein applied to the gel was the same for each of the methods. The major effect of washing with different buffers is seen in zone C; these bands are caseins and were identified as previously described [11]. As a whole,

TABLE II

THE APPARENT MOLECULAR MASSES OF MILK FAT GLOBULE MEMBRANE PROTEINS (kDa)

Band	Coomassie blue stain	Periodate-Schiff stain
A_1	226.0	–
A_2	–	167.5
A_3	142.4	– [a]
A_4	128.0	133.2
A_5	–	107.2
B_1	74.5	74.5
B_2	64.4	62.8
B_3	49.5	48.6
B_4	46.0	45.8
B_5	43.5	–
C_1	35.7	35.4
C_2	32.9	32.4
C_3	32.0	31.3
C_4	30.3	–
C_5	29.2	29.0

[a] This region is faintly positive for carbohydrate in some preparations but not in others.



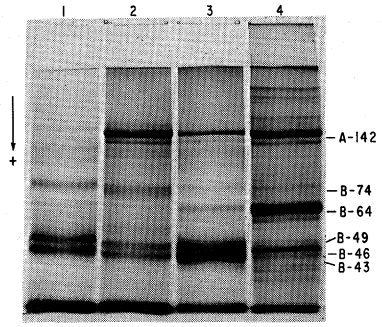


Fig. 1. (Left.) SDS-polyacrylamide gel electrophoresis of proteins from the milk fat globule membranes prepared as described in text. The stacking and separating gels contained 4% and 15% acrylamide, respectively. Proteins were detected by Coomassie Blue. S, standard purified proteins (phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.3 kDa); 1–6 represent, respectively, the methods of preparation described in Table I. The same fractions were also electrophoresed and stained for carbohydrate. Only ovalbumin (43 kDa) in low molecular mass standards and catalase (60 kDa) and ferritin (220 kDa) in high molecular mass standards were stained with periodate-Schiff reagent. A summary of the molecular masses obtained is given in Table II.

Fig. 2. (Right.) SDS-polyacrylamide gel electrophoresis of the major proteins of the milk fat globule membrane. The stacking and separating gels contained 4% and 8.5% acrylamide, respectively. (1) Water-soluble fraction; (2) 0.02 M NaCl-soluble fraction; (3) 0.6 M NaCl-soluble fraction; and (4) insoluble fraction.

Fig. 1 illustrates that as the salt concentration in the buffers decreases, the milk proteins (particularly the caseins, zone C) are not washed out.

Table II shows the apparent molecular masses of the milk fat globule membrane proteins as determined from Coomassie blue-stained (Fig. 1) and periodate-Schiff-stained gels. In order to determine the molecular masses of the proteins in zone A, the Pharmacia High Molecular Weight Calibration mixture, which included thyroglobulin (330 000) and ferritin (220 000 for 1/2 unit), was applied to Laemmli gels. In the carbohydrate gel, only ovalbumin, catalase and ferritin were stained, but their R_F values were nearly identical to those determined in the protein-stained gels. Therefore, the same R_F values of the standard proteins were applied to both gels (Table II).

Preparation and salt fractionation of fat globule membrane proteins

Delipidated fat globule membrane proteins were prepared according to the method of Herald and Brunner [6]. This procedure uses cold 35% ethanol/ethyl ether to dissociate the lipid-protein complexes of the fat globule membrane and has been used extensively in the literature [18]. Gel

electrophoresis of the materials obtained during each step in the method of Herald and Brunner indicated that the milk proteins, especially the caseins (C-region, Fig. 1) which are not initially removed by water washing, were removed by the delipidation procedure. The bands common to all preparations (the A and B regions) remained in the delipidated sample.

The delipidated fat globule membrane proteins were extracted successively using deionized water followed by 0.02 M and 0.6 M NaCl. Approx. 60% of the protein recovered is in the insoluble fraction and 36% in the 0.6 M NaCl-soluble fraction. The remaining 4% is recovered in the water-soluble and the 0.02 M NaCl soluble fractions. This fractionation was carried out to reconcile the method of Jackson and co-workers [19] and of Butler and Oskvig [20] who extracted with water only, with that of Brunner and Herald [6] who extracted with 0.02 M NaCl. Proteins extracted by the stepwise procedure are designated S, while those not extracted are termed insoluble (I). The electrophoretic patterns of three soluble fractions and the insoluble fraction of delipidated fat globule membrane proteins are shown in Fig. 2. Since the major bands of interest are found in the A and B regions, 8.5% acrylamide concentration was selected over 15% acrylamide, yielding better resolution of the high (A-region) and intermediate molecular weight (B-region) protein fractions. All of the C region bands merge at the front (compare Figs. 1 and 2). Water-soluble fraction (S) (slot 1) contained two proteins in approximately equal amounts (B-49 and B-46). There is a trace of protein in zone A and a considerable amount of protein B-74. The 0.02 M NaCl soluble fraction (slot 2) is slightly more complex and has a good deal of the A-142 band relative to the water soluble fraction. The 0.6 M NaCl soluble fraction (slot 3) contained the B-49, B-46 and B-43 proteins and a considerable amount of A-142. The insoluble (I) fraction (slot 4) has four major bands heavily stained with Coomassie blue; they are designated as I-A-142, I-B-64, I-B-49 and I-B-46. A companion gel was stained for glycoproteins (periodate-Schiff stain for carbohydrate). All of the proteins in the B region stained positively with both Coomassie blue and periodate-Schiff. In the A region, the I-A-142 was stained with Coomassie blue and only faintly with periodate-Schiff in some large-scale preparations but not in others. Two others were positvely stained for carbohydrate only (I-A-167 and 107).

Gel chromatography of protein fractions

Chromatography of the three soluble fractions as well as the insoluble fraction of delipidated fat globule membrane proteins was carried out in the presence of SDS as previously described [15] on a Sephacryl S-200 column. The 0.02 M NaCl fraction contains a unique peak eluting near the void volume. After rechromatography this protein was identified as the periodate-Schiff negative component of A-142. No resolution of the major bands from the insoluble fraction was achieved. Repeated chromatography seemed to indicate an interaction between several of the B components even in the presence of detergents and mercaptoethanol.

Isolation of protein components by preparative gel electrophoresis

Because of the resolution achieved on polyacrylamide gel electrophoresis, it was decided to excise the bands from gels in order to isolate proteins for further study. Two types of commercial apparatus for extraction and concentra-

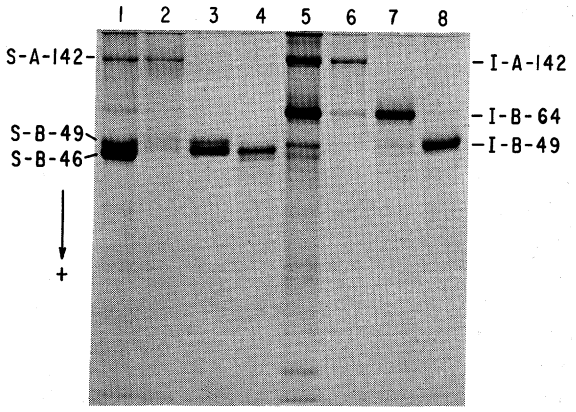


Fig. 3. SDS gel patterns for proteins of 0.6 M NaCl-soluble and insoluble fractions recovered from the preparative gels. The stacking and separating gels contained 4% and 15% acrylamide, respectively. (1) 0.6 M NaCl soluble fraction; (2) S-A-142; (3) mixture of S-B-49 and S-B-46; (4) S-B-46; (5) insoluble fraction; (6) I-A-142; (7) I-B-64; and (8) I-B-49.

TABLE III

COMPARISON OF AMINO ACID CONTENTS OF THE PROTEIN ISOLATED FROM 0.02 M NaCl-SOLUBLE FRACTION AND PURIFIED XANTHINE OXIDASE

Cystine and tryptophan were not determined by this analysis.

Amino acid	Mol%	
	0.02 M NaCl soluble fraction xanthine oxidase	purified xanthine oxidase [21]
Asp	9.10	9.00
Thr	6.91	7.38
Ser	4.67	6.39
Glu	10.74	10.44
Pro	5.73	5.39
Gly	7.20	8.59
Ala	7.58	8.00
Val	9.14	6.96
Met	0.59	1.10
Ile	5.27	5.41
Leu	9.82	9.36
Tyr	2.92	2.05
Phe	5.58	5.22
Lys	7.41	7.13
His	2.37	2.52
Arg	4.89	5.07

tion of protein from gel were used. They gave unsatisfactory results, since there was either high contamination or low recovery of proteins. To alleviate these problems, a simplified apparatus for gel elution was put together and a modified slot former used (see Materials and Methods).

Fig. 3 shows the results of electrophoretic analysis of 0.6 M NaCl soluble (S) fraction and insoluble (I) fraction from which the proteins were extracted, as well as the bands which were excised from the gel, recovered by elution, and re-electrophoresed. The S-B-49 and S-B-46 bands were also prepared in the same way from the water and 0.02 M NaCl soluble fractions. Slots 3 and 4 illustrate the upper and lower halves of the S-B-49 and S-B-46 regions, respectively. These two components were never completely resolved. Slots 6, 7 and 8 show I-A-142, I-B-64 and I-B-49 bands at levels with the proteins of the insoluble fraction (slot 5).

Amino acid analysis of isolated milk fat globule membrane proteins

As judged by Fig. 3, good purification of the

TABLE IV

AMINO ACID ANALYSIS OF FOUR ISOLATED BANDS OF SOLUBLE AND INSOLUBLE FRACTIONS

Cystine and tryptophan were not determined by this analysis. Values represent averages of two or three complete experiments, comparing the two methods of electrophoresis.

Amino acid	Mol%						
	S-A-142	I-A-142		I-B-64		I-B-49	
	Laemmli gel	Cyanogum gel	Laemmli gel	Cyanogum gel	Laemmli gel	Cyanogum gel	Laemmli gel
Asp	8.09	8.73	9.06	8.23	8.75	9.99	9.22
Thr	5.16	5.94	5.88	4.55	4.70	6.82	5.56
Ser	17.83	11.49	14.36	10.41	13.87	13.02	13.10
Glu	13.07	12.72	12.44	13.20	13.62	13.97	12.97
Pro	2.18	4.83	4.33	5.22	4.68	5.41	3.73
Gly	17.28	12.09	13.62	10.92	13.45	12.29	12.96
Ala	7.82	7.26	7.58	6.28	7.08	7.40	7.47
Val	6.15	6.16	5.89	6.18	5.81	4.33	6.32
Met	0.52	1.27	0.84	1.50	1.00	0.41	1.35
Ile	3.53	4.17	3.97	4.79	3.84	3.74	3.65
Leu	5.18	7.96	6.89	8.29	7.16	6.71	7.60
Tyr	2.77	2.61	2.74	3.17	2.74	2.59	3.06
Phe	3.12	3.92	3.51	4.85	3.60	2.92	2.85
Lys	3.65	5.07	4.59	4.68	4.20	4.29	4.93
His	2.25	2.73	1.93	3.29	1.78	2.92	1.96
Arg	2.16	3.70	3.37	4.94	4.44	3.18	3.79

selected bands was achieved, and sufficient weights of materials recovered so that amino acid analysis was attempted. The amino acid composition of the protein, which was chromatographically isolated from the A region of the 0.02 M NaCl-soluble fraction was compared (Table III) with that of purified xanthine oxidase [21] using the procedure of Cornish-Bowden [22]. By this method unrelated proteins will show an $S\Delta n$ value of more than $0.93N$, while strongly related proteins will show values less than $0.42N$. The two proteins gave an $S\Delta n$ value of $0.72N$, which shows a weak indication of compositional relatedness. The amino acid compositions of two other proteins isolated from the same region by gel extraction are shown in Table IV. $S\Delta n$ values were calculated for the various fractions of A-142 region. Except for the relationship noted above, no two A-142 proteins are related ($S\Delta n > 2$). Additionally, in three large-scale preparations from different herds, the I-A-142 fraction stained positively for carbohydrate in one instance, very faintly in a second, and not at all in the third. This indicates that the A-142 region is quite complex and may contain several proteins, only one of which is xanthine oxidase. The results here confirm those of Mather et al. [23] who showed two different Triton-X-soluble proteins in the 142 kDa region by isoelectric focusing.

All protein fractions in Table IV show an elevated content of three amino acids (serine, glutamic acid and glycine). Walker et al. [24] reported that the most persistent contaminants for amino acid analysis of gel extracted proteins are Tris and glycine. Tris [24] elutes in the vicinity of histidine; in our experiments, an unknown peak which emerged between histidine and lysine but it did not interfere with determination of these amino acids. Walker et al. [24] also found that glycine in the electrolyte buffer caused a higher glycine content, while higher serine contents seemed to result from the buffer or from the polyacrylamide gels during electrophoresis and extraction processes. Even though the extracted proteins were dialyzed in Spectrapor dialysis bags for several days, these three amino acids were not removed completely. Summation of the μmoles recovered after hydrolysis indicated that a significant amount of non-proteinaceous material was recovered in many instances. Therefore, two electrophoretic methods were compared. One was the discontinuous method reported by Laemmli [12] as exemplified in Figs. 1 and 2, and the other was the continuous SDS-polyacrylamide gel electrophoresis as previously described [15] using cyanogum. The SDS-polyacrylamide gel using the cyanogum reduced the contaminants somewhat, since this method did not require glycine in the system; however, band resolution was poorer [15]. In both methods, the recoveries of half-cystine were too low to be quantitated. Using the $S\Delta n$ procedure, the values for the same molecular weight fractions obtained by the two different electrophoretic methods (Cyanogum vs. Laemmli) were $0.85N$, $0.77N$ and $0.32N$ for I-A-142, I-B-64, and I-B-49, respectively. The $S\Delta n$ values showed that there are apparently some differences between the two methods for higher molecular weight proteins. This is significant, since $S\Delta n$, in theory converges at higher molecular weights.

The amino acid composition data given in Table IV were subjected to further statistical analyses to determine whether or not the variations observed are due either to the electrophoretic method or to actual differences in amino acid content. When the data are grouped with regard to protein fractions, and analyzed amino acid by amino acid, all except Val, Tyr and His vary significantly (92% confidence limit or greater) from fraction to fraction (see Appendix I). When the two different methods of isolation are compared, only four amino acids vary significantly by method (Ala, Phe, Lys and Ile). Thus, at first glance, it would appear that the individual proteins are different from each other regardless of the extraction method. However, when the data are considered as a whole, a significant statistical interaction term was observed for seven amino acids. This indicates that the method of electrophoresis influences the recovery of individual amino acids in some protein fractions more so than in others (particularly, according to $S\Delta n$, with the high molecular weight fractions). Thus, comparisons among fractions should be reliably made only with proteins prepared by the same method. The Tris-glycine discontinuous system of Laemmli was selected because it has extremely high resolving power which technically permits a finer excision of protein

bands. This results in a lower total acrylamide carry over when used in conjunction with single slot, preparative gels and the modified elution device. The results from these studies proved to be statistically reliable. Studies on bovine serum albumin gave amino acid compositional data very close to those published in the literature [18].

The amino acid analysis of fractions which contain nearly equal mixtures of 46–49 kDa bands and appear in every soluble fraction (S-B-49 and 46 in all soluble fractions) are shown in Table V. Differences in amino acid composition were compared among these fractions and the I-B-49 fraction by the procedure of Cornish-Bowden [22]. Mixtures of S-B-49 and 46 proteins which were isolated from all three soluble fractions differed by $0.2N$ or less, while the I-B-49 protein is different from all three ($S\Delta n > 1.2$). Despite the problems associated with gel extraction, the composition of these water-soluble proteins closely resembles glycoprotein B which was previously reported on in detail [15].

Although it has been suggested that the $S\Delta n$ method is valid only for comparing proteins of similar molecular mass, when employing the cautions given by Cornish-Bowden [22], some conclusions can be drawn about proteins of unequal molecular weight. Considering all of the protein fractions discussed above regardless of molecular weight and comparing compositions for those prepared by the same procedure, only three 'cross' molecular weight comparisons pass the strong test ($0.42N$ or less). I-A-142 differed from I-B-64 and I-B-49 by only $0.16N$ and $0.15N$; I-B-64 differed from I-B-49 by $0.12N$. The extremely low $S\Delta n$ values among I-A-142, I-B-64 and I-B-49 showed these proteins to be very similar despite the fact that the three isolated fractions had different mobilities on the Laemmli gel, have some significant differences in many amino acids (Tables IV and V), and that the I-A-142 band shows high variability in carbohydrate staining. Therefore, these proteins may possibly be structurally related.

Amino acid compositions have thus been obtained for the major bands of milk fat globule membrane which are visualized by Coomassie blue and present independent of the method of preparation of the membranes. Band A-142 contains at least three proteins of similar molecular mass, one of which is xanthine oxidase. The A region also contains a major glycoprotein (167 kDa) which is unstained by Coomassie blue and is not dansylated; this component was therefore not isolated in this study. Band I-B-64 is not extracted from the membrane by simple aqueous solvents and is closely related to I-A-142 and I-B-49; this band is most likely the milk fat globule membrane component recently termed butyrophilin by Franke and co-workers [25]. Bands B-49 and 46 are also heterogeneous and contain glycoproteins soluble in aqueous media (S-B-49 and S-B-46), as well as an insoluble glycoprotein(s) I-B-49 which is more related to I-B-64 and I-A-142 than to the soluble glycoproteins. The soluble glycoproteins are quite distinct from the insoluble components and have been previously characterized as glycoprotein B [15] and referred to elsewhere as BAMP [19,20], and bands 15–16 [4].

TABLE V

AMINO ACID COMPOSITIONS OF 46–49 kDa PROTEINS OF MILK FAT GLOBULE MEMBRANE

Cystine and tryptophan were not determined by this analysis. Average of two or three complete experiments.

Amino acid	Mol%		
	Water-soluble fraction S-B-49 and 46	0.02 M NaCl-soluble fraction S-B-49 and 46	0.6 M NaCl-soluble fraction S-B-49 and 46
Asp	11.62	11.11	10.81
Thr	8.55	7.26	6.97
Ser	6.65	8.04	7.22
Glu	10.88	10.77	11.16
Pro	4.69	4.10	4.61
Gly	10.47	11.15	10.34
Ala	6.04	7.03	6.56
Val	4.41	5.05	4.61
Met	1.31	1.23	1.59
Ile	5.59	5.53	6.71
Leu	8.01	8.54	8.32
Tyr	3.93	3.35	3.75
Phe	4.92	4.57	4.94
Lys	4.39	4.94	4.29
His	3.96	2.53	3.43
Arg	4.58	4.78	4.68

Appendix I

Statistical analysis of amino acid composition data from Table IV

Amino acid	Probability > F [a]		
	Protein fraction	Method of extraction	Interaction term
Asp	0.0002	0.8083	0.0362
Thr	0.0001	0.4127	0.0107
Ser	0.0692	0.3890	0.4973
Glu	0.0805	0.5715	0.0454
Pro	0.0692	0.3890	0.4973
Gly	0.0157	0.6900	0.5591
Ala	0.0007	0.0010	0.1845
Val	0.9223	0.1777	0.3514
Met	0.0871	0.1493	0.0030
Ile	0.0017	0.0012	0.0159
Leu	0.0014	0.9263	0.0181
Tyr	0.7802	0.5670	0.3516
Phe	0.0158	0.0844	0.4552
Lys	0.0075	0.0413	0.0645
His	0.8913	0.1777	0.8756
Arg	0.0010	0.7167	0.5903

[a] Probability of a larger observed value of F under the test hypothesis that the compositions are not different due to the listed variable; given amino acids are significantly different when the tabular values are less than or equal to 0.08 (e.g., underlined values in last two columns).

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